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14. ABSTRACT
Primary cilia (PC) are solitary, sensory organelles that are critical for several signaling pathways. The purpose of this project is to determine the function of PC in breast cancer. First, we demonstrated that PC are more frequent in interlobular fibroblasts and myoepithelial cells than luminal cells in normal human breast. Of 26 breast cancers, rare PC were identified in cancer epithelial cells in only 1 cancer, which was of the triple negative subtype. In 11 breast cancer cell lines, PC were present at low frequency in 4, but were absent in the remainder. The cancer lines with PC were of the basal B subtype, analogous to the clinical triple negative subtype. Our data indicate a decrease of PC in breast cancer and an association of PC with the basal B subtype. Next, we developed mouse models with deletion of PC in epithelial or myoepithelial cells of the mammary gland for evaluation of the function of PC in development and cancer. We confirmed that *Ift88* was deleted in the mammary gland of Cre-positive mice and that PC were disrupted. Nevertheless, minimal disruption to normal mammary development was observed. Studies to determine the role of PC in tumor progression are ongoing.

15. SUBJECT TERMS
primary cilia, breast cancer, tumor samples, mouse models

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Table of Contents

Page

Introduction.
Primary cilia (PC) are common organelles present on nearly all cells and are involved in transmitting both chemical and mechanical signals (Satir and Christensen, 2007). The process of Intraflagellar Transport (IFT) is responsible for building and maintaining the structure and function of cilia. The absence of *Ift88/Tg737/Polaris*, a core molecular component of the IFT machinery, results in the loss of cilia (Haycraft et al., 2007; Yoder et al., 2002). The role of PC in various disease processes has only recently been appreciated. Still nothing is known about the role of PC in normal mammary development or tumor formation. The purpose of this synergistic study was to begin to address the role of this understudied yet potentially important organelle in cancer. We proposed two specific aims. The first was to characterize the incidence of cilia in normal and malignant breast samples and to then determine the role of cilia in proliferation, apoptosis, clonogenicity, growth in soft agar, migration and invasion in human tumor cell lines. The second aim was to characterize a mouse model for the role of PC in normal mammary gland development and in tumor progression. This report summarizes progress during the first year of the study.

Body.
The part of the study detailing the incidence of primary cilia in normal and invasive breast tissue (Task #3) has been completed and a manuscript describing the results is in revision. The results are detailed in the partnering PI' s progress report (Dr. Andra Frost) and briefly summarized here. The incidence of PC in normal breast tissue and 26 breast cancer samples as well as on epithelial cell lines derived from normal breast and 11 breast cancer cell lines was determined. PC were detected by immunoflourescent staining for alpha acetylated tubulin (Poole et al., 2001); AAT; Sigma, clone 6-11B-1). Analysis of the incidence of PC in normal tissue indicated that PC were most frequent in interlobular fibroblasts and basal (myoepithelial) cells and less frequent on luminal and alveolar type epithelial cells. PC were identified at very low frequency on epithelial cells from only 1 cancer, which was of the triple negative subtype. The results suggested that PC were more frequent in normal breast epithelium than in breast cancer. PC were more frequent in epithelial cells derived from benign breast, but were present at a low frequency in 4 of 11 cancer cell lines and were absent in the remaining 7. The 4 cancer lines that demonstrated PC were all of the basal B subtype, which is analogous to the clinical triple negative subtype described above. The results demonstrate that primary cilia are more frequent on cells lines derived from benign breast than on cells derived from breast cancer and suggest that the incidence of PC decreases in the progression of normal to cancerous epithelial cells.

The initiating PI, Dr. Rosa Serra, was primarily responsible for characterizing the mouse models of PC in mammary development and cancer described in Tasks 4 through 6. The goal of Task 4 was to determine if PC are required for normal mammary development. To address this question we used a Cre/Lox strategy to delete *Ift88* and thus PC in mammary epithelium. Mice in which exons 4 through 6 of the *Ift88* locus were flanked with loxP sites were generated previously (Haycraft et al., 2007). When crossed to mouse lines expressing Cre recombinase the deletion was shown to result in a null allele and depletion of cilia in the specific targeted cells. Though a series of crosses, MMTVCre; *Ift88*loxP/loxP mice were generated. The MMTV promoter/enhancer is the most common promoter used to drive gene expression in the mammary glands of transgenic mice (Wagner et al., 2001). It preferentially directs gene expression to adult mammary epithelium. Deletion of the *Ift88* gene was confirmed using a PCR based assay that

simultaneously detects the loxP, wild type, and deleted alleles of *Ift88* from DNA extracted from epithelial cells isolated from control (Cre-negative littermates) and MMTV-Cre;*Ift88*loxP/loxP glands (Figure 1 Lanes 1,2; (Johnson et al., 2008). Almost all of the loxP allele was deleted as demonstrated by the ratio of the PCR products for the loxP and deleted alleles (top and bottom bands respectively). This result suggests that Cre is active in virtually all of the mammary epithelial cells. Loss of PC in the mammary gland was also confirmed by immunoflourescent staining and confocal microscopy of AAT, to mark the PC, and gamma tubulin, to mark the basal body (Figure 2). In this case, sections from glands of pregnant mice were used because there is more epithelium in each section making it easier to identify and measure any decrease in PC staining that may be present in the mutants. In the pregnant controls, PC were detected by the red AAT staining and green gamma tubulin staining. Many cilia were observed with a characteristic "comet" structure: the green gamma tubulin at one end and then the red "tail" of the cilia extending outward (Figure 2A insert). In sections from mutant glands, green gamma tubulin and red AAT staining were evident; however, the characteristic "comet" structures indicative of PC were not clearly visible. Instead green and red dots could be seen adjacent to or slightly overlapping each other (Figure 2B insert). The results indicated that the PC were disrupted in the MMTV Cre mutant glands. Further quantification of these results is underway. Next, glands were harvested from control and MMTVCre;*Ift88*loxP/loxP mice at varying stages of development and whole mount carmine staining of the glands was performed (Figure 3). Branching and structure of control and mutant glands were comparable at 5 (not shown) and 12 weeks of age (Figure 3A,B). Only minor differences were detected in control and mutant glands in 12.5 dpc (not shown) and 14.5 dpc (Figure 3C-F) pregnant glands. Although alveolar structures were clearly present in the mutant glands, the structures appeared smaller than controls (Figure 3E,F). Additional quantification of this result is underway. Nevertheless, the mutant mice were able to lactate and support normal healthy litters indicating that the loss of cilia did not affect the primary function of the mammary gland.

While we were doing these experiments, Dr. Frost completed her analysis of the incidence of PC on varying cell types within normal human breast tissue. She found that the incidence of PC on luminal epithelial cells is quite low and that most of the PC in the epithelial compartment of the mammary gland reside on myoepithelial cells. To address this issue directly, we obtained the K14Cre mouse line from Jackson labs (Dassule et al., 2000). K14 has been shown to target myoepithelial cells in the mammary gland (Kuraguchi et al., 2009). After a series of crosses, K14Cre; *Ift88*loxP/loxP mice were generated. Deletion of the *Ift88* gene was confirmed using the PCR method described above (Figure 1 Lanes 3,4). The deleted allele was detected in DNA isolated from whole mammary gland (epithelium and stroma) indicating the Cre is active in the mammary gland. The low level of overall deletion is expected in this case since myoepithelial cells represent only a small fraction of the cells within the entire gland. We next confirmed loss of cilia in the myoepithelial cells using immunoflourescent staining and standard wide field microscopy for AAT (Figure 2 C,D) and by double staining with AAT and smooth muscle actin, to define myoepithelial cells (not shown). PC were easily detected in the basal (myoepithelial) layer of cells along the ducts of control glands. In contrast, we were not able to detect PC in the basal cell layer of mutant glands although PC staining was still observed in the stroma (Figure 2D). We have also started to characterize the phenotype of these mice using carmine whole mount staining. So far we have looked at glands from control and mutant mice at 5 weeks (not

shown) and at 9 weeks of age (Figure 3G,H). At 9 weeks, a potential slight reduction in the level of branching was observed but additional quantification of these results is required.

Task #5 is to determine if loss of PC is sufficient to induce tumor formation in mice. If PC act as tumor suppressors it is possible that loss of PC would be sufficient to induce tumor formation even in the absence of a developmental phenotype. We are still in the process of generating control and MMTV Cre;*Ift88* mutant mice for this task.

Task #6 is to determine if loss of PC affect tumor formation in cooperation with an oncogene. Most tumors arise though a combination of mutations. Loss of PC may cooperate with other oncogenic factors to promote (or reduce) tumor formation. We have not started the crosses for this task yet because we felt that since the mice had a minimal developmental phenotype we had to confirm that *Ift88* was deleted and that PC were in fact disrupted before we could start breeding large numbers of mice (see task #4). Since we now know that PC are disrupted and that the MMTVCre*Ift88*loxP/loxP males and females can breed normally and females can lactate we can use a more efficient breeding scheme to generate these mice. In addition, we plan to switch to the MMTV PyVmT model, which has a shorter tumor latency time than the MMTV-neu mice (50% by 3 months versus 50% by 7 months) but still generate adenocarcinomas with similar pathology to human tumors (Guy et al., 1992a; Guy et al., 1992b).

Key accomplishments.
- Generated mice with targeted deletion of *Ift88* and disruption of PC in mammary epithelial cells via MMTV Cre and in myoepithelial cells specifically using K14 Cre.
-Demonstrated that the Cre is active in the mammary gland and that PC are disrupted.
-Shown that disruption f PC has minimal effect on development of the mammary gland and that mice with disruption of PC can lactate and support pups.

Reportable outcomes.
1- Invited review submitted: Yuan K, Serra, R, Frost AR. Primary Cilia in the Breast and Breast Cancer. The Open Breast Journal, Invited review, submitted, 2010.

2- Research paper in revision: Yuan K, Frolova N, Xie Y, Wang D, Cook L, Kwon Y-J, Steg AD, Serra R, and Frost AR. Primary Cilia Are Decreased in Breast Cancer: Analysis of a Collection of Human Breast Cancer Cell Lines and Tissues. In Revision Journal of Histochemistry and Cytochemistry, 2010.

3- Platform presentation, annual meeting of the American Association for Cancer Research, 2009.

Conclusion.
In the first year of this synergistic study we have begun to address the role of the PC in normal mammary development and breast cancer.

The data from the partnering PI suggest that the incidence of PC decreases in the progression of normal to cancerous epithelial cells. Furthermore, analysis of the incidence of PC in normal tissue indicated that PC are most frequent in interlobular fibroblasts and basal (myoepithelial)

cells and less frequent on luminal and alveolar type epithelial cells. The results support our hypothesis that PC may act as tumor suppressors.

To better test this hypothesis, we generated mice using Cre/lox technology with targeted deletion of *Ift88*, a protein required for IFT and formation of PC. We have confirmed that *Ift88* is deleted in the mammary gland of Cre-positive mice and that cilia are disrupted. Nevertheless, there is minimal disruption to normal mammary development and the mice lactate and support their pups. Studies to determine the role of PC in tumor progression are ongoing.

Understanding the role of cilia in tumor progression will provide novel insights into the molecular mechanisms of cancer and provide additional targets for rational therapies.

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Appendix.

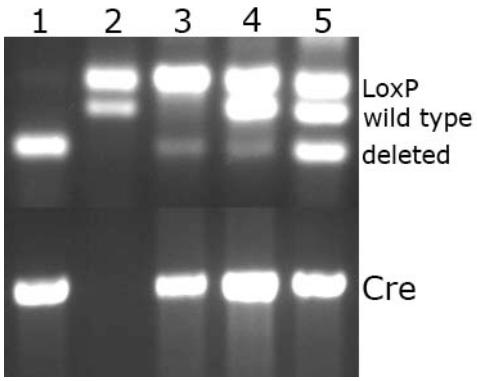

Figure 1. PCR assay for *Ift88* deletion. Lane 1- DNA extracted from epithelial cells isolated from MMTVCre+;Ift88loxP/loxP mammary glands. Lane 2- DNA extracted from epithelial cells isolated from MMTVCre-negative;Ift88loxP/wt mammary glands. Lane 3- DNA extracted from from total K14Cre+;Ift88loxP/loxP mammary glands. Lane 4-DNA extracted from from total K14Cre+;Ift88loxP/wt mammary glands. Lane 5- Control DNA from the tail of a K14Cre+;Ift88loxP/wt mouse. PCR products: LoxP = Ift88loxP allele, wild type = the wild type allele, deleted = the deleted Ift88 allele. Cre = Cre transgene.

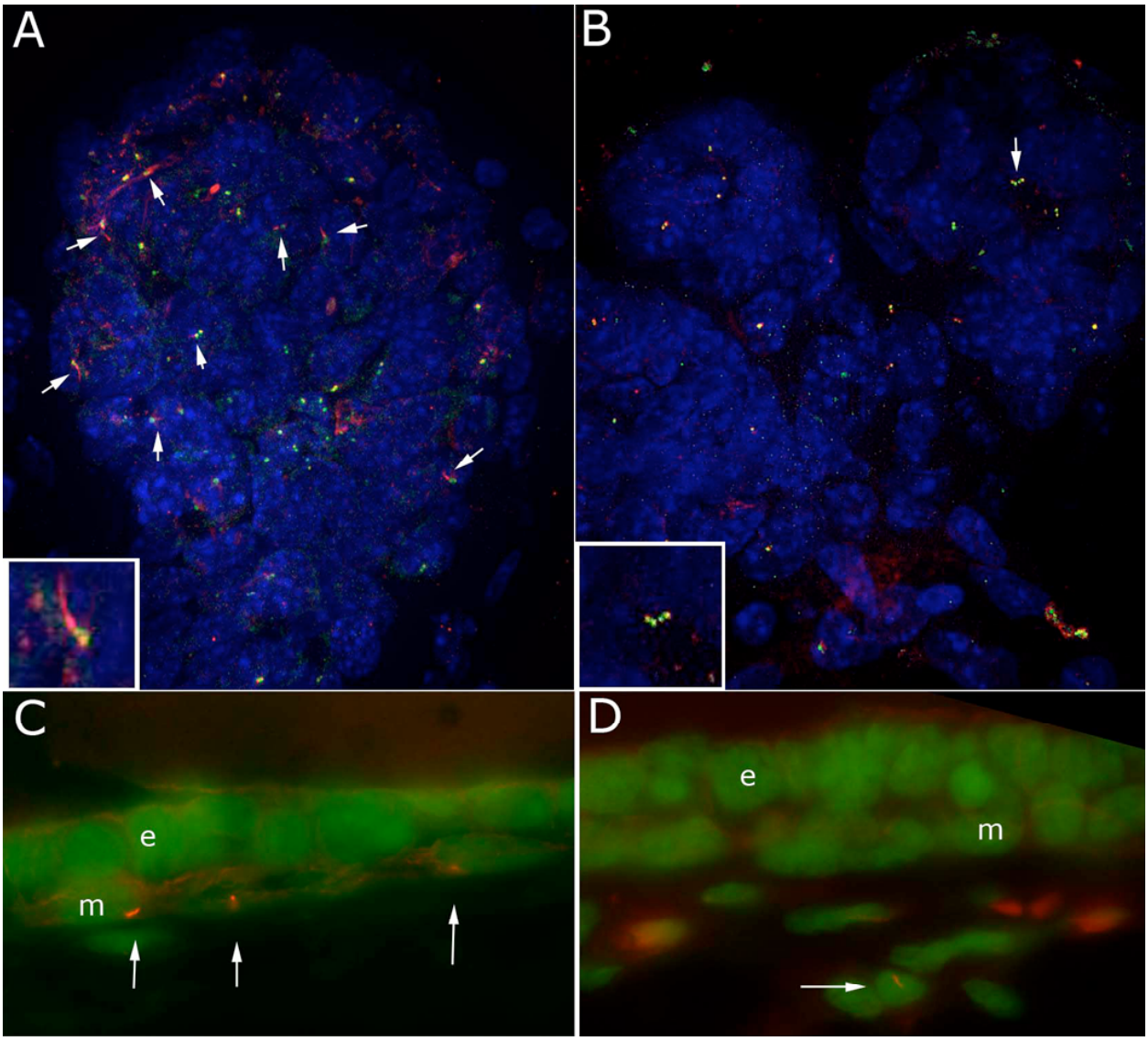

Figure 2. Cilia staining. Sections from 12.5 dpc pregnant control (A) and MMTVCre+;*Ift88*loxP/loxP (B) mammary glands were immuno stained for AAT (red), gamma tubulin (green) and nuclei (Dapi, blue) and observed using confocal microscopy. PC demonstrated a "comet" appearance in control sections (insert A) with green staining at one end representing the basal body and a red "tail" representing the cilium. These structures were not observed in sections from mutant glands. Instead only small dots of red and green were seen together (insert B). Sections from 8 week old quiescent control (C) and K14Cre; *Ift88*loxP/loxP (D) glands were immunostained for AAT (red) and nuclei (YoPro, green) and observed using wide field microscopy. PC were seen as red staining in the myoepithelial cells of the ducts in control sections (C). We were not able to find PC in the ducts of the mutant mice (D); however, PC were detected on cells in the stroma. Arrows = examples of PC.

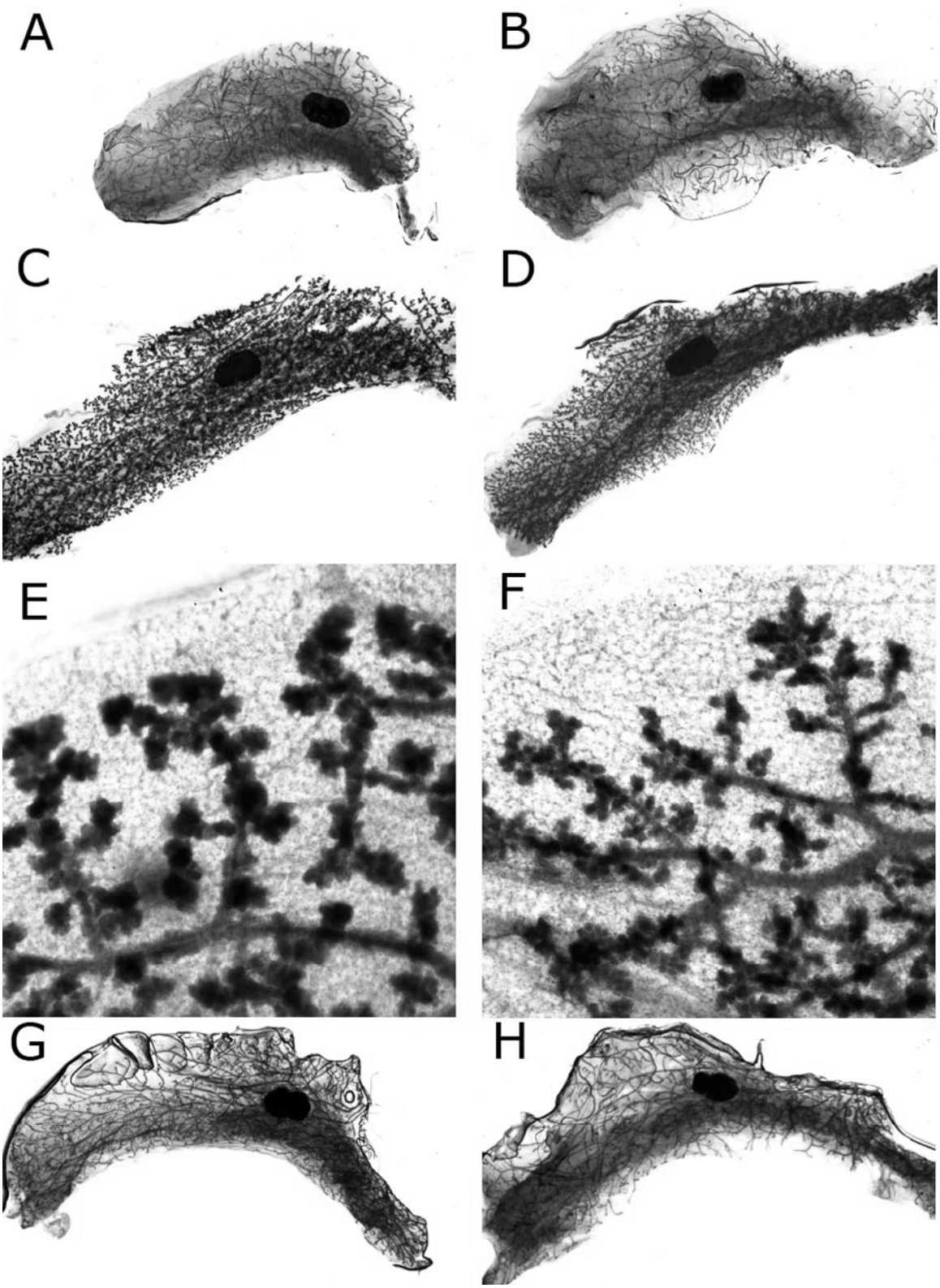

Figure 3. Whole mount mammary staining. Mammary glands were isolated from control A,C,E,G and MMTVCre+;*Ift88*loxP/loxP (B,D,F) and K14Cre+;*Ift88*loxP/loxP (H) mice at 12 weeks quiescent (A,B), 12.5 dpc pregnant (C-F), and 9 weeks quiescent (G,H).